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(54) Title: A DNA CONSTRUCT ENCODING THE YAP3 SIGNAL PEPTIDE (57) Abstract A DNA construct comprising the following sequence: 5'-P-SP-(LP) _n -PS-HP-3' wherein P is a promoter sequence, SP is a DNA sequence encoding the yeast aspartic protease 3 (YAP3) signal peptide, LP is a DNA sequence encoding a leader peptide, n is 0 or 1, PS is a DNA sequence encoding a peptide defining a yeast processing site, and HP is a DNA sequence encoding a polypeptide which is heterologous to a selected host organism. The YAP3 signal peptide provides efficient secretion of heterologous proteins in yeast.		

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A DNA CONSTRUCT ENCODING THE YAP3 SIGNAL PEPTIDE

FIELD OF INVENTION

The present invention relates to a DNA construct comprising the YAP3 signal peptide for secretion of a heterologous polypeptide, a yeast cell containing the DNA construct and a method of producing heterologous polypeptides in yeast from the DNA construct.

BACKGROUND OF THE INVENTION

Yeast organisms produce a number of proteins which are synthesized intracellularly, but which have a function outside the cell. Such extracellular proteins are referred to as secreted proteins. These secreted proteins are expressed initially inside the cell in a precursor or a pre-protein form containing a presequence ensuring effective direction of the expressed product across the membrane of the endoplasmic reticulum (ER). The presequence, normally named a signal peptide, is cleaved off from the rest of the protein during translocation. Once entered in the secretory pathway, the protein is transported to the Golgi apparatus. From the Golgi the protein can follow different routes that lead to compartments such as the cell vacuole or the cell membrane, or it can be routed out of the cell to be secreted to the external medium (Pfeffer, S.R. and Rothman, J.E. Ann.Rev.Biochem. 56 (1987), 829-852).

Several approaches have been suggested for the expression and secretion in yeast of proteins heterologous to yeast. European published patent application No. 88 632 describes a process by which proteins heterologous to yeast are expressed, processed and secreted by transforming a yeast organism with an expression vehicle harbouring DNA encoding the desired protein and a signal peptide, preparing a culture of the transformed organism, growing the culture and recovering the protein from

the culture medium. The signal peptide may be the signal peptide of the desired protein itself, a heterologous signal peptide or a hybrid of native and heterologous signal peptide.

A problem encountered with the use of signal peptides heterologous to yeast might be that the heterologous signal peptide does not ensure efficient translocation and/or cleavage after the signal peptide.

The S. cerevisiae MFa1 (α -factor) is synthesized as a prepro form of 165 amino acids comprising signal-or prepeptide of 19 amino acids followed by a "leader" or propeptide of 64 amino acids, encompassing three N-linked glycosylation sites followed by (LysArg(Asp/Glu, Ala)_{2,3} α -factor)₄ (Kurjan, J. and Herskowitz, I. Cell 30 (1982), 933-943). The signal-leader part of the preproMFa1 has been widely employed to obtain synthesis and secretion of heterologous proteins in S. cerevisiae.

Use of signal/leader peptides homologous to yeast is known from i.a. US patent specification No. 4,546,082, European published patent applications Nos. 116 201, 123 294, 123 544, 163 529, and 123 289 and DK patent application No. 3614/83.

In EP 123 289 utilization of the S. cerevisiae α -factor precursor is described whereas WO 84/01153 indicates utilization of the Saccharomyces cerevisiae invertase signal peptide and DK 3614/83 utilization of the Saccharomyces cerevisiae PH05 signal peptide for secretion of foreign proteins.

US patent specification No. 4,546,082, EP 16 201, 123 294, 123 544, and 163 529 describe processes by which the α -factor signal-leader from Saccharomyces cerevisiae (MFa1 or MFa2) is utilized in the secretion process of expressed heterologous proteins in yeast. By fusing a DNA sequence encoding the S. cerevisiae MFa1 signal/leader sequence at the 5' end of the gene for the desired protein secretion and processing of the desired protein was demonstrated.

A number of secreted proteins are routed so as to be exposed to a proteolytic processing system which can cleave the peptide bond at the carboxy end of two consecutive basic amino acids. This enzymatic activity is in S. cerevisiae encoded by the KEX 2 gene (Julius, D.A. et al., Cell 37 (1984b), 1075). Processing of the product by the KEX 2 gene product is needed for the secretion of active S. cerevisiae mating factor α (M α or α -factor) but is not involved in the secretion of active S. cerevisiae mating factor a.

- 10 The use of the mouse salivary amylase signal peptide (or a mutant thereof) to provide secretion of heterologous proteins expressed in yeast has been described in WO 89/02463 and WO 90/10075. It is the object of the present invention to provide a more efficient expression and/or secretion in yeast of
15 heterologous proteins.

SUMMARY OF THE INVENTION

It has surprisingly been found that the signal peptide of the yeast aspartic protease 3 is capable of providing improved secretion of proteins expressed in yeast compared to the mouse
20 salivary amylase signal peptide.

Accordingly, the present invention relates to a DNA construct comprising the following sequence

5'-P-SP-(LP)_n-PS-HP-3'

wherein

- 25 P is a promoter sequence,
SP is a DNA sequence encoding the yeast aspartic protease 3 (YAP3) signal peptide,
LP is a DNA sequence encoding a leader peptide,
n is 0 or 1,

PS is a DNA sequence encoding a peptide defining a yeast processing site, and

HP is a DNA sequence encoding a polypeptide which is heterologous to a selected host organism.

5 The term "signal peptide" is understood to mean a presequence which is predominantly hydrophobic in nature and present as an N-terminal sequence of the precursor form of an extracellular protein expressed in yeast. The function of the signal peptide is to allow the heterologous protein to be secreted to enter
10 the endoplasmic reticulum. The signal peptide is cleaved off in the course of this process. The YAP3 signal sequence has been reported previously, fused to its native gene (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137. A DNA construct wherein the YAP3 signal sequence is fused to a DNA sequence
15 encoding a heterologous polypeptide is believed to be novel. The YAP3 signal peptide has not previously been reported to provide efficient secretion of heterologous polypeptides in yeast.

In the present context, the expression "leader peptide" is
20 understood to indicate a peptide whose function is to allow the heterologous polypeptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the medium, (i.e. export of the expressed polypeptide across the cell wall or at least through
25 the cellular membrane into the periplasmic space of the cell).

The expression "heterologous polypeptide" is intended to indicate a polypeptide which is not produced by the host yeast organism in nature.

In another aspect, the present invention relates to a
30 recombinant expression vector comprising the DNA construct of the invention.

In a further aspect, the present invention relates to a cell transformed with the recombinant expression vector of the invention.

In a still further aspect, the present invention relates to a method of producing a heterologous polypeptide, the method comprising culturing a cell which is capable of expressing a heterologous polypeptide and which is transformed with a DNA construct of the invention in a suitable medium to obtain expression and secretion of the heterologous polypeptide, after which the heterologous polypeptide is recovered from the medium.

DETAILED DESCRIPTION OF THE INVENTION

In a specific embodiment, the YAP3 signal peptide is encoded by the following DNA sequence

15 ATG AAA CTG AAA ACT GTA AGA TCT GCG GTC CTT TCG TCA CTC TTT GCA
TCT CAG GTC CTT GGC (SEQ ID No:1)

or a suitable modification thereof encoding a peptide with a high degree of homology (at least 60%, more preferably at least 70%, sequence identity) to the YAP3 signal peptide. Examples of suitable modifications" are nucleotide substitutions which do not give rise to another amino acid sequence of the peptide, but which may correspond to the codon usage of the yeast organism into which the DNA sequence is introduced, or nucleotide substitutions which do give rise to a different amino acid sequence of the peptide (although the amino acid sequence should not be modified to the extent that it is no longer able to function as a signal peptide). Other examples of possible modifications are insertion of three or multiples of three nucleotides at either end of or within the sequence, or deletion of three or multiples of three nucleotides at either end of or within the sequence.

In the sequence 5'-P-SP-(LP)_n-PS-HP-3', n is preferably 1. In other words, although the YAP3 signal peptide may, in some instances, in itself provide secretion and/or processing of the heterologous polypeptide, a leader or pro-peptide sequence is preferably present. The leader may be a yeast MFα1 leader peptide or a synthetic leader peptide, e.g. one of the leader peptides disclosed in WO 89/02463 or WO 92/11378 or a derivative thereof capable of effecting secretion of a heterologous polypeptide in yeast. The term "synthetic" is intended to indicate that the leader peptides in question are not found in nature. Synthetic yeast leader peptides may, for instance be constructed according to the procedures described in WO 89/02463 or WO 92/11378.

The yeast processing site encoded by the DNA sequence PS may suitably be any paired combination of Lys and Arg, such as Lys-Arg, Arg-Lys, Lys-Lys or Arg-Arg, which permits processing of the heterologous polypeptide by the KEX2 protease of Saccharomyces cerevisiae or the equivalent protease in other yeast species (D.A. Julius et al., Cell 37, 1984, 1075 ff.). If KEX2 processing is not convenient, e.g. if it would lead to cleavage of the polypeptide product, a processing site for another protease may be selected instead comprising an amino acid combination which is not found in the polypeptide product, e.g. the processing site for FX₀, Ile-Glu-Gly-Arg (cf. Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).

The heterologous protein produced by the method of the invention may be any protein which may advantageously be produced in yeast. Examples of such proteins are aprotinin, tissue factor pathway inhibitor or other protease inhibitors, insulin or insulin precursors, human or bovine growth hormone, interleukin, glucagon, tissue plasminogen activator, transforming growth factor α or β, platelet-derived growth factor, enzymes, or a functional analogue thereof. In the present context, the term "functional analogue" is meant to

indicate a polypeptide with a similar function as the native protein (this is intended to be understood as relating to the nature rather than the level of biological activity of the native protein). The polypeptide may be structurally similar to
5 the native protein and may be derived from the native protein by addition of one or more amino acids to either or both the C- and N-terminal end of the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more amino
10 acids at either or both ends of the native protein or at one or several sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence. Such modifications are well known for several of the proteins mentioned above.

15 The DNA construct of the invention may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp.
20 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned into the yeast expression vector. It should be noted that the sequence 5'-P-SP-(LP)_n-PS-HP-3' need not be prepared in a single
25 operation, but may be assembled from two or more oligonucleotides prepared synthetically in this fashion.

One or more parts of the DNA sequence 5'-P-SP-(LP)_n-PS-HP-3' may also be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA
30 sequences coding for said parts (typically HP) by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989). In this case, a genomic or cDNA sequence encoding
35 a signal peptide may be joined to a genomic or cDNA sequence

encoding the heterologous protein, after which the DNA sequence may be modified by the insertion of synthetic oligonucleotides encoding the sequence 5'-P-SP-(LP)_n-PS-HP-3' in accordance with well-known procedures.

- 5 Finally, the DNA sequence 5'-P-SP-(LP)_n-PS-HP-3' may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by annealing fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA
10 sequence, in accordance with standard techniques. Thus, it may be envisaged that the DNA sequence encoding the signal peptide or the heterologous polypeptide may be of genomic or cDNA origin, while the sequence 5'-P-SP-(LP)_n-PS may be prepared synthetically.
- 15 The recombinant expression vector carrying the sequence 5'-P-SP-(LP)_n-PS-HP-3' may be any vector which is capable of replicating in yeast organisms. In the vector, the promoter sequence (P) may be any DNA sequence which shows transcriptional activity in yeast and may be derived from genes
20 encoding proteins either homologous or heterologous to yeast. The promoter is preferably derived from a gene encoding a protein homologous to yeast. Examples of suitable promoters are the Saccharomyces cerevisiae MFα1, TPI, ADH I, ADH II or PGK promoters, or corresponding promoters from other yeast species,
25 e.g. Schizosaccharomyces pombe. Examples of suitable promoters are described by, for instance, Russell and Hall, J. Biol. Chem. 258, 1983, pp. 143-149; Russell, Nature 301, 1983, pp. 167-169; Ammerer, Meth. Enzymol. 101, 1983, pp. 192-201; Russell et al., J. Biol. Chem. 258, 1983, pp. 2674-2682;
30 Hitzeman et al, J. Biol. Chem. 225, 1980, pp. 12073-12080; Kawasaki and Fraenkel, Biochem. Biophys. Res. Comm. 108, 1982, and T. Alber and G. Kawasaki, J. Mol. Appl. Genet. 1, 1982, pp. 419-434.

The sequences indicated above should also be operably connected to a suitable terminator, e.g. the TPI terminator (cf. T. Alber and G. Kawasaki, J. Mol. Appl. Genet. 1, 1982, pp. 419-434), or the yeast CYC1 terminator.

- 5 The recombinant expression vector of the invention further comprises a DNA sequence enabling the vector to replicate in yeast. Examples of such sequences are the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. the Schizo-
10 saccharomyces pombe TPI gene as described by P.R. Russell, Gene 40, 1985, pp. 125-130, or the yeast URA3 gene.

The procedures used to insert the sequence 5'-P-SP-(LP)_n-PS-HP-3' into a suitable yeast vector containing the information necessary for yeast replication, are well known to persons
15 skilled in the art (cf., for instance, Sambrook, Fritsch and Maniatis, op.cit.). It will be understood that the vector may be constructed either by first preparing a DNA construct containing the entire sequence and subsequently inserting this fragment into a suitable expression vector, or by sequentially
20 inserting DNA fragments containing genetic information for the individual elements (such as the promoter sequence, the signal sequence, the leader sequence, or DNA coding for the heterologous polypeptide) followed by ligation.

The yeast organism transformed with the vector of the invention
25 may be any suitable yeast organism which, on cultivation, produces large amounts of the heterologous polypeptide in question. Examples of suitable yeast organisms may be strains of Saccharomyces, such as Saccharomyces cerevisiae, Saccharomyces kluyveri, or Saccharomyces uvarum,
30 Schizosaccharomyces, such as Schizosaccharomyces pombe, Kluyveromyces, such as Kluyveromyces lactis, Yarrowia, such as Yarrowia lipolytica, or Hansenula, such as Hansenula polymorpha. The transformation of the yeast cells may for

instance be effected by protoplast formation followed by transformation in a manner known per se.

The medium used to cultivate the cells may be any conventional medium suitable for growing yeast organisms. The secreted
5 heterologous protein, a significant proportion of which will be present in the medium in correctly processed form, may be recovered from the medium by conventional procedures including separating the yeast cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the
10 supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

BRIEF DESCRIPTION OF THE DRAWINGS

15 The invention is further described in the following examples with reference to the appended drawings wherein

Fig. 1A and 1B schematically show the construction of plasmid pLaC257;

Fig. 2 shows the DNA sequence and derived amino acid sequence
20 of the EcoRI-XbaI insert in pLaC257 (SEQ ID No:2);

Fig. 3A and 3B schematically show the construction of plasmid pLaC242Apr;

Fig. 4 shows the DNA sequence and derived amino acid sequence of the EcoRI-XbaI fragment of pAPRScl, wherein the protein
25 sequence shown in italics is derived from the random expression cloned DNA fragment (SEQ ID No:4);

Fig. 5 schematically shows the construction of plasmid pLaC263;

Fig. 6 shows the DNA sequence and derived amino acid sequence of the EcoRI-XbaI fragment of pLaC263 (SEQ ID No:6);

Fig. 7A and 7B show the DNA sequence and derived amino acid sequence of human tissue factor pathway inhibitor (TFPI) including its native signal peptide (SEQ ID No:8)

Fig. 8A shows the DNA sequence and derived amino acid sequence of the spx3 signal peptide and 212 leader peptide (shown in WO 89/02463) N-terminally fused to the TFPI sequence in plasmid pYES-212 TFPI161-117Q (SEQ ID No:10);

Fig. 8B shows the DNA sequence and derived amino acid sequence of the YAP3 signal peptide and 212 leader peptide N-terminally fused to the TFPI sequence in plasmid pYES-yk TFPI161-117Q (SEQ ID No:12); and

Fig. 9 shows restriction maps of plasmids pYES21, pP-212TFPI161-117Q; pYES-212TFPI161-117Q and pYES-ykTFPI161-117Q.

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLES

Plasmids and DNA materials

All expression plasmids contain 2 μ DNA sequences for replication in yeast and use either the S. cerevisiae URA3 gene or the Schizosaccharomyces pombe triose phosphate isomerase gene (POT) as selectable markers in yeast. POT plasmids are described in EP patent application No. 171 142. A plasmid containing the POT-gene is available from a deposited E. coli strain (ATCC 39685). The POT plasmids furthermore contain the S. cerevisiae triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pMT742 (M. Egel-Mitani et al., Gene 73, 1988, pp. 113-120) (see fig. 1) except for the region defined by the Sph-XbaI restriction sites encompassing the P_{TPI} and the coding region for

signal/leader/product. The URA3 plasmide use P_{TPI} and the iso-I-cytochrome C terminator (T_{cyc1}).

The P_{TPI} has been modified with respect to the sequence found in pMT742, only in order to facilitate construction work. An internal SphI restriction site has been eliminated by SphI cleavage, removal of single stranded tails and religation. Furthermore, DNA sequences, upstream to and without any impact on the promoter, have been removed by Bal31 exonuclease treatment followed by addition of an SphI restriction site linker. This promoter construction present on a 373 bp SphI-EcoRI fragment is designated $P_{TPI\delta}$ and when used in plasmids already described this promoter modification is indicated by the addition of a δ to the plasmid name.

Finally a number of synthetic DNA fragments have been employed all of which were synthesized on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (S.L. Beaucage and M.H. Caruthers (1981) Tetrahedron Letters 22, 1859-1869). The oligonucleotides were purified by polyacrylamide gel electrophoresis under denaturing conditions. Prior to annealing complementary pairs of such DNA single strands these were kinased by T4 polynucleotide kinase and ATP.

All other methods and materials used are common state of the art knowledge (J. Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press) Cold Spring Harbor, N.Y. 1989).

Example 1

The modified mouse salivary amylase signal peptide ($MSA3_{sp}$) (described in WO 89/02463) of the expression cassette of plasmid pLSC6315D3 (described in Example 3 of WO 92/11378) which contains a DNA sequence coding for the insulin precursor

MI3 (B(1-29)-Ala-Ala-Lys-A(1-21)), was replaced with the YAP3 signal peptide in the following steps:

A construct for easy exchange of signal peptides was made. Through site-directed mutagenesis an Asp718 site was introduced 5 just prior to the signal initiation codon in pLaC1966 (cf. WO 89/02463, fig. 5), by the double primer method applying a mutagenic primer NOR494:

3'-ATTTGCTGCCATGGTACTTTTCAGAAGG (SEQ ID No:14)

where bold letters indicate mutations and the underlined 10 sequence indicates the initiation codon.

The resulting plasmid was termed pLaC1966-Asp718 (see Fig. 1).

The nucleotide sequence of the region covering the junction between signal peptide and leader peptide of the expression cassette in pLSC6315D3 was modified, by replacing the Apal- 15 HgiAI restriction fragment with a synthetic DNA stretch, NOR 2521/2522:

NOR2521: 5'-CAA CCA ATA GAC ACG CGT AAA GAA GGC CTA
CAG CAT GAT TAC GAT ACA GAG ATC TTG GAG (SEQ
ID No:15)

20 NOR2522: 5'-C CAA GAT CTC TGT ATC GTA ATC ATG CTG TAG
GCC TTC TTT ACG CGT GTC TAT TGG TTG GGC C (SEQ
ID No:16)

The resulting plasmid was termed pLSC6315D3R (see Fig. 1).

The SphI-Asp718 fragment of pLaC1966-Asp718 was ligated with 25 SphI-MluI cut pLSC6315D3R plasmid and a synthetic stretch of DNA encoding the YAP3 signal peptide:

YAP-spl: 5'-GT ACC AAA ATA ATG AAA CTG AAA ACT GTA AGA

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TCT GCG GTC CTT TCG TCA CTC TTT GCA TCT CAG
GTC CTT GGC CAA CCA ATA GAC A (SEQ ID No:17)

YAP-sp2: 5'-CG CGT GTC TAT TGG TTG GCC AAG GAC CTG AGA TGC
AAA GAG TGA CGA AAG GAC CGC AGA TCT TAC
5 AGT TTT CAG TTT CTA TAT TTT G (SEQ ID No:18)

The resulting plasmid pLaC257 essentially consists of pLSC6315D3, in which the MSA3 signal peptide has been replaced by the YAP3 signal peptide (see Fig. 2).

Yeast transformation: S. cerevisiae strain MT663 (E2-7B XE11-36
10 a/a, Δtpi/Δtpi, pep 4-3/pep 4-3) (the yeast strain MT663 was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen in connection with filing WO 92/11378 and was given the deposit number DSM 6278) was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to
15 an O.D. at 600 nm of 0.6.

100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of a solution containing 1.2 M sorbitol, 25 mM Na₂EDTA pH = 8.0 and 6.7 mg/ml dithiotreitol. The suspension was incubated at 30°C
20 for 15 minutes, centrifuged and the cells resuspended in 10 ml of a solution containing 1.2 M sorbitol, 10 mM Na₂EDTA, 0.1 M sodium citrate, pH 0 5.8, and 2 mg Novozym®234. The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and 10 ml of
25 CAS (1.2 M sorbitol, 10 mM CaCl₂, 10 mM Tris HCl (Tris = Tris(hydroxymethyl)aminomethane) pH = 7.5) and resuspended in 2 ml of CAS. For transformation, 1 ml of CAS-suspended cells was mixed with approx. 0.1 μg of plasmid pLaC257 and left at room temperature for 15 minutes. 1 ml of (20% polyethylene
30 glycol 4000, 20 mM CaCl₂, 10 mM CaCl₂, 10 mM Tris HCl, pH = 7.5) was added and the mixture left for a further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7

mM CaCl_2 , 14 $\mu\text{g/ml}$ leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. Then, 6 ml of top agar (the SC medium of Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory (1982)) containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium.

Transformant colonies were picked after 3 days at 30°C, 10 reisolated and used to start liquid cultures. One transformant was selected for further characterization.

Fermentation: Yeast strain MT663 transformed with plasmid pLaC257 was grown on YPD medium (1% yeast extract, 2% peptone (from Difco Laboratories), and 3% glucose). A 1 liter culture 15 of the strain was shaken at 30°C to an optical density at 650 nm of 24. After centrifugation the supernatant was isolated.

MT663 cells transformed with plasmid pLSC6315D3 and cultured as described above were used for a comparison of yields of MI3 insulin precursor. Yields of MI3 were determined directly on 20 culture supernatants by the method of Snel, Damgaard and Mollerup, Chromatographia 24, 1987, pp. 329-332. The results are shown below.

	plasmid	MI3 yield
25	pSLC63.15D3 (Msa3 _{sp})	100%
	pLaC257 (YAP3)	120%

Example 2

Plasmid pLSC6315D3 was modified in two steps. First, the MSA3 signal peptide was replaced by the spx3 signal peptide by exchanging the SphI-ApaI fragment with the analogous fragment

from pLaC212spx3 (cf. WO 89/02463). From the resulting plasmid pSLC63.15spx3, a 302bp EcoRI-DdeI fragment was isolated and fused to the 204 bp NcoI-XbaI fragment of pKFN1003 (WO 90/10075) containing the DNA sequence encoding aprotinin via a 5 synthetic linker DNA, NOR2101/2100 (see Fig. 3)

NOR2101: 5'-T AAC GTC GC (SEQ ID No:19)

NOR2100: 5'-CAT GGC GAC G (SEQ ID No:20)

The resulting plasmid, pLaC242-Apr (see Fig. 3), was cleaved with ClaI, dephosphorylated and applied in cloning of random 10 5'-CG-overhang fragments of DNA isolated from *S. cerevisiae* strain MT663, according to the description in WO 92/11378. Transformation and fermentation of yeast strain MT663 was carried out as described in Example 1.

From the resulting library yeast transformants harbouring the 15 plasmid pAPR-ScI (prepared by the method described in WO 92/11378) containing a leader the sequence of which is given in Fig. 4, was selected by screening. The spx3 signal peptide of pAPR-ScI was replaced by the YAP3 signal peptide by fusing the SphI-StyI fragment from pLaC257 with the 300 bp NheI-XbaI 20 fragment of pAPR-ScI via the synthetic linker DNA MH1338/1339 (see Fig. 5):

MH 1338: 5'-CTT GGC CAA CCA TCG AAA TTG AAA CCA G (SEQ ID No:21)

25 MH 1339: 5'-CT AGC TGG TTT CAA TTT CGA TGG TTG GC (SEQ ID No:22)

The resulting plasmid was termed pLaC263 (see Fig. 5). The DNA sequence and derived amino acid sequence of the EcoRI-XbaI fragment of pLaC263 appears from Fig. 6.

17

plasmid	aprotinin yield
pAPR-Scl (Spx3 _{sp})	100%
pLaC263	136%

Example 3

5 A synthetic gene coding for human TFPI, the DNA sequence of which was derived from the published sequence of a cDNA coding for human tissue factor pathway inhibitor (TFPI) (Wun et al., J. Biol. Chem. 263 (1988) 6001-6004), was prepared by step-wise cloning of synthetic restriction fragments into plasmid pBS(+).
10 The resulting gene was contained on a 928 base pair (bp) SalI restriction fragment. The gene had 26 silent nucleotide substitutions in degenerate codons as compared to the cDNA resulting in fourteen unique restriction endonuclease sites. The DNA sequence of the 928 bp SalI fragment and the
15 corresponding amino acid sequence of human TFPI (pre-form) is shown in Fig. 7 (SEQ ID No:8).

This DNA sequence was subsequently truncated to code for a TFPI variant composed of the first 161 amino acids. A non-glycosylated variant, TFPI₁₋₁₆₁-117Gln in which the AAT-codon for
20 Asn117 was replaced by CAA coding for Gln was constructed by site-directed mutagenesis in a manner known per se using synthetic oligonucleotides. The DNA sequence encoding TFPI₁₋₁₆₁-117Gln was preceded by the synthetic signal-leader sequence 212spx3 (cf. WO 89/02463), see Fig. 8A. This construction was
25 inserted into the plasmid pP-212TFPI161-117Q (based on a vector of the POT-type (G. Kawasaki and L. Bell, US patent 4,931,373), cf. Fig. 8).

A 1.1 kb SphI-XbaI fragment containing the coding region for 212spx3-TFPI₁₋₁₆₁-117Gln was isolated and cloned into the plasmid
30 pYES21 derived from the commercially available (Stratagene) vector pYES2.0 (cf. Fig. 8). This plasmid contains 2μ sequence

for replication in yeast, the yeast URA3 gene for plasmid selection in ura3 strains, the β -lactamase gene for selection in E. coli, the ColE1 origin of replication for replication in E. coli, the f1 origin for recovery of single-stranded DNA 5 plasmid from superinfected E. coli strains, and the yeast CYC1 transcriptional terminator. The SphI-XbaI fragment was cloned into pYES 2.0 in front of the CYC1 terminator. The resulting plasmid pYES-212TFPI161-117Q (cf. Fig. 9) was cleaved with PflMI and EcoRI to remove the coding region for the mouse 10 salivary amylase signal peptide which was replaced by a double-stranded synthetic oligonucleotide sequence coding for the YAP3 signal peptide:

MHJ 1131 5'AAT TCA AAC TAA AAA ATG AAG CTT AAA ACT GTA AGA
TCT GCG GTC CTT TCG TCA CTC TTT GCA TCG CAG GTC CTA GGT CAA CCA
15 GTC A (SEQ ID No:23)

MHJ 1132 5'CTG GTT GAC CTA GGA CCT GCG ATG CAA AGA GTG ACG
AAA GGA CCG CAG ATC TTA CAG TTT TAA GCT TCA TTT TTT AGT TTG
(SEQ ID No:24)

resulting in plasmid pYES-ykTFPI161-117Q (cf. Fig. 8B and Fig. 20 9).

Plasmids pYES-212TFPI161-117Q and pYES-ykTFPI161-117Q were transformed into the haploid yeast strain YNG318 (MAT α ura3-52 leu2-42 pep4-41 his4-539 [cir⁺]). Plasmid selection was for Ura⁺ cells. Reisolated transformants were grown in 50 ml of 25 synthetic complete medium lacking uracil (SC-ura) for 3 days at 30°C. After measuring cell density (OD₆₀₀), the cultures were centrifuged and the resulting supernatants were analysed for the level of secreted FXa/TF/FVIIa-dependent chromogenic TFPI-activity (P.M. Sandset et al., Thromb.Res. 47, 1987, pp. 389-30 400). The mean activity measured for supernatants from strains containing plasmid pYES-212TFPI161-117Q (i.e. the plasmid

containing the mouse salivary amylase signal sequence) was 0.65 U/ml•OD. The mean activity measured for supernatants from strains containing plasmid pYES-ykTFPI161-117Q was 1.00 U/ml•OD.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- 5 (A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): 2880
10 (G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: A DNA Construct Encoding the YAP3 Signal Peptide

(iii) NUMBER OF SEQUENCES: 24

15 (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

20 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- 30 (A) ORGANISM: *Saccharomyces cerevisiae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGAAACTGA AACTGTAAAG ATCTGGGGTC CTTTGTGCAC TCTTTGCATC TCAGGTCTT 60
GGC 63

(2) INFORMATION FOR SEQ ID NO: 2:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 476 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

21

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 81..452

10 (ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 81..293

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

15 (B) LOCATION: 294..452

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

	GAATTCATTC AAGAAATAGTT CAAACAAGAA GATTACAAAC TATCAATTTC ATACACAATA	60
	TAAACGACGG TACCAAAATA ATG AAA CTG AAA ACT GTA AGA TCT GCG GTC	110
	Met Lys Leu Lys Thr Val Arg Ser Ala Val	
20	-71 -70 -65	
	CTT TCG TCA CTC TTT GCA TCT CAG GTC CTT GGC CAA CCA ATA GAC ACG	158
	Leu Ser Ser Leu Phe Ala Ser Gln Val Leu Gly Gln Pro Ile Asp Thr	
	-60 -55 -50	
	CGT AAA GAA GGC CTA CAG CAT GAT TAC GAT ACA GAG ATC TTG GAG CAC	206
25	Arg Lys Glu Gly Leu Gln His Asp Tyr Asp Thr Glu Ile Leu Glu His	
	-45 -40 -35 -30	
	ATT GGA AGC GAT GAG TTA ATT TTG AAT GAA GAG TAT GTT ATT GAA AGA	254
	Ile Gly Ser Asp Glu Leu Ile Leu Asn Glu Glu Tyr Val Ile Glu Arg	
	-25 -20 -15	
30	ACT TTG CAA GCC ATC GAT AAC ACC ACT TTG GCT AAG AGA TTC GTT AAC	302
	Thr Leu Gln Ala Ile Asp Asn Thr Thr Leu Ala Lys Arg Phe Val Asn	
	-10 -5 1	
	CAA CAC TTG TGC GGT TCC CAC TTG GTT GAA GCT TTG TAC TTG GTT TGC	350
35	Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys	
	5 10 15	
	GGT GAA AGA GGT TTC TTC TAC ACT CCT AAG GCT GCT AAG GGT ATT GTC	398
	Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ala Ala Lys Gly Ile Val	
	20 25 30 35	

22

GAA CAA TGC TGT ACC TOC ATC TGC TOC TTG TAC CAA TTG GAA AAC TAC 446
 Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr
 40 45 50

TGC AAC TAGAOGCAGC COGCAGGCTC TAGA 476
 5 Cys Asn

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 124 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Lys Leu Lys Thr Val Arg Ser Ala Val Leu Ser Ser Leu Phe Ala
 15 -71 -70 -65 -60

Ser Gln Val Leu Gly Gln Pro Ile Asp Thr Arg Lys Glu Gly Leu Gln
 -55 -50 -45 -40

His Asp Tyr Asp Thr Glu Ile Leu Glu His Ile Gly Ser Asp Glu Leu
 -35 -30 -25

20 Ile Leu Asn Glu Glu Tyr Val Ile Glu Arg Thr Leu Gln Ala Ile Asp
 -20 -15 -10

Asn Thr Thr Leu Ala Lys Arg Phe Val Asn Gln His Leu Cys Gly Ser
 -5 1 5

His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe
 25 10 15 20 25

Tyr Thr Pro Lys Ala Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser
 30 35 40

Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
 45 50

30 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 450 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(ix) FEATURE:

5

(A) NAME/KEY: CDS

(B) LOCATION: 76..441

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 76..267

10

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 268..441

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

	GAATTCATTC AAGAATAGTT CAAACAAGAA GATTACAAAC TATCAATTTT ATACACAATA	60
15	TAAACGATTAA AAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC GGA	111
	Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly	
	-64 -60 -55	
	TTC TGC TGG GCC CAA CCA TCG AAA TTG AAA CCA GCT AGC GAT ATA CAA	159
20	Phe Cys Trp Ala Gln Pro Ser Lys Leu Lys Pro Ala Ser Asp Ile Gln	
	-50 -45 -40	
	ATT CTT TAC GAC CAT GGT GTG AGG GAG TTC GGG GAA AAC TAT GTT CAA	207
	Ile Leu Tyr Asp His Gly Val Arg Glu Phe Gly Glu Asn Tyr Val Gln	
	-35 -30 -25	
	GAG TTG ATC GAT AAC ACC ACT TTG GCT AAC GTC GCC ATG GCT GAG AGA	255
25	Glu Leu Ile Asp Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu Arg	
	-20 -15 -10 -5	
	TTG GAG AAG AGA AGG CCT GAT TTC TGT TTG GAA CCT CCA TAC ACT GGT	303
	Leu Glu Lys Arg Arg Pro Asp Phe Cys Leu Glu Pro Pro Tyr Thr Gly	
	1 5 10	
30	CCA TGT AAA GCT AGA ATC ATC AGA TAC TTC TAC AAC GCC AAG GCT GGT	351
	Pro Cys Lys Ala Arg Ile Ile Arg Tyr Phe Tyr Asn Ala Lys Ala Gly	
	15 20 25	
	TTG TGT CAA ACT TTC GTT TAC GGT GGC TGC AGA GCT AAG AGA AAC AAC	399
35	Leu Cys Gln Thr Phe Val Tyr Gly Gly Cys Arg Ala Lys Arg Asn Asn	
	30 35 40	
	TTC AAG TCT GCT GAA GAC TGC ATG AGA ACT TGT GGT GGT GCC	441
	Phe Lys Ser Ala Glu Asp Cys Met Arg Thr Cys Gly Gly Ala	
	45 50 55	
	TAATCTAGA	450

24

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 122 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 -64 -60 -55 -50

10 Gln Pro Ser Lys Leu Lys Pro Ala Ser Asp Ile Gln Ile Leu Tyr Asp
 -45 -40 -35

His Gly Val Arg Glu Phe Gly Glu Asn Tyr Val Gln Glu Leu Ile Asp
 -30 -25 -20

15 Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu Arg Leu Glu Lys Arg
 -15 -10 -5

Arg Pro Asp Phe Cys Leu Glu Pro Pro Tyr Thr Gly Pro Cys Lys Ala
 1 5 10 15

Arg Ile Ile Arg Tyr Phe Tyr Asn Ala Lys Ala Gly Leu Cys Gln Thr
 20 25 30

20 Phe Val Tyr Gly Gly Cys Arg Ala Lys Arg Asn Asn Phe Lys Ser Ala
 35 40 45

Glu Asp Cys Met Arg Thr Cys Gly Gly Ala
 50 55

(2) INFORMATION FOR SEQ ID NO: 6:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 470 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

35 (ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 81..461

25

(ix) FEATURE:

(A) NAME/KEY: sig_peptide
(B) LOCATION: 81..287

(ix) FEATURE:

5 (A) NAME/KEY: mat_peptide
(B) LOCATION: 288..461

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```

GAATTCATTC AAGAAATAGTT CAAACAAGAA GATTACAAAC TATCAATTTC ATACACAATA      60
TAAAGGACGG TACCAAAATA ATG AAA CTG AAA ACT GTA AGA TCT GCG GTC      110
10      Met Lys Leu Lys Thr Val Arg Ser Ala Val
           -69                -65                -60

CTT TCG TCA CTC TTT GCA TCT CAG GTC CTT GGC CAA CCA TCG AAA TTG      158
Leu Ser Ser Leu Phe Ala Ser Gln Val Leu Gly Gln Pro Ser Lys Leu
           -55                -50                -45

15 AAA CCA GCT AGC GAT ATA CAA ATT CTT TAC GAC CAT GGT GTG AGG GAG      206
Lys Pro Ala Ser Asp Ile Gln Ile Leu Tyr Asp His Gly Val Arg Glu
           -40                -35                -30

TTC GGG GAA AAC TAT GTT CAA GAG TTG ATC GAT AAC ACC ACT TTG GCT      254
20      Phe Gly Glu Asn Tyr Val Gln Glu Leu Ile Asp Asn Thr Thr Leu Ala
           -25                -20                -15

AAC GTC GGC ATG GCT GAG AGA TTG GAG AAG AGA AGG OCT GAT TTC TGT      302
Asn Val Ala Met Ala Glu Arg Leu Glu Lys Arg Arg Pro Asp Phe Cys
           -10                -5                1                5

TTG GAA OCT CCA TAC ACT GGT CCA TGT AAA GCT AGA ATC ATC AGA TAC      350
25      Leu Glu Pro Pro Tyr Thr Gly Pro Cys Lys Ala Arg Ile Ile Arg Tyr
           10                15                20

TTC TAC AAC GCC AAG GCT GGT TTG TGT CAA ACT TTC GTT TAC GGT GGC      398
Phe Tyr Asn Ala Lys Ala Gly Leu Cys Gln Thr Phe Val Tyr Gly Gly
           25                30                35

30 TGC AGA GCT AAG AGA AAC AAC TTC AAG TCT GCT GAA GAC TGC ATG AGA      446
Cys Arg Ala Lys Arg Asn Asn Phe Lys Ser Ala Glu Asp Cys Met Arg
           40                45                50

ACT TGT GGT GGT GCC TAATCTAGA      470
35      Thr Cys Gly Gly Ala
           55

```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 127 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

26

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Lys Leu Lys Thr Val Arg Ser Ala Val Leu Ser Ser Leu Phe Ala
 -69 -65 -60 -55

5 Ser Gln Val Leu Gly Gln Pro Ser Lys Leu Lys Pro Ala Ser Asp Ile
 -50 -45 -40

Gln Ile Leu Tyr Asp His Gly Val Arg Glu Phe Gly Glu Asn Tyr Val
 -35 -30 -25

10 Gln Glu Leu Ile Asp Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu
 -20 -15 -10

Arg Leu Glu Lys Arg Arg Pro Asp Phe Cys Leu Glu Pro Pro Tyr Thr
 -5 1 5 10

Gly Pro Cys Lys Ala Arg Ile Ile Arg Tyr Phe Tyr Asn Ala Lys Ala
 15 20 25

15 Gly Leu Cys Gln Thr Phe Val Tyr Gly Gly Cys Arg Ala Lys Arg Asn
 30 35 40

Asn Phe Lys Ser Ala Glu Asp Cys Met Arg Thr Cys Gly Gly Ala
 45 50 55

(2) INFORMATION FOR SEQ ID NO: 8:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 928 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens
- 30 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 8..919
- (ix) FEATURE:
 (A) NAME/KEY: sig_peptide
 (B) LOCATION: 8..91
- 35 (ix) FEATURE:
 (A) NAME/KEY: mat_peptide
 (B) LOCATION: 92..919

	GTCACC	ATG	ATT	TAC	ACA	ATG	AAG	AAA	GTA	CAT	GCA	CTT	TGG	GCT	AGC	49	
		Met	Ile	Tyr	Thr	Met	Lys	Lys	Val	His	Ala	Leu	Trp	Ala	Ser		
		-28			-25				-20						-15		
5	GTA	TGC	CTG	CTG	CTT	AAT	CTT	GCC	OCT	GCC	OCT	CTT	AAT	GCT	GAT	TCT	97
	Val	Cys	Leu	Leu	Leu	Asn	Leu	Ala	Pro	Ala	Pro	Leu	Asn	Ala	Asp	Ser	
				-10					-5						1		
	GAG	GAA	GAT	GAA	GAA	CAC	ACA	ATT	ATC	ACA	GAT	ACG	GAG	CTC	CCA	CCA	145
10	Glu	Glu	Asp	Glu	Glu	His	Thr	Ile	Ile	Thr	Asp	Thr	Glu	Leu	Pro	Pro	
		5						10					15				
	CTG	AAA	CTT	ATG	CAT	TCA	TTT	TGT	GCA	TTC	AAG	GCG	GAT	GAT	GGG	CCC	193
	Leu	Lys	Leu	Met	His	Ser	Phe	Cys	Ala	Phe	Lys	Ala	Asp	Asp	Gly	Pro	
		20					25					30					
	TGT	AAA	GCA	ATC	ATG	AAA	AGA	TTT	TTC	TTC	AAT	ATT	TTC	ACT	CGA	CAG	241
15	Cys	Lys	Ala	Ile	Met	Lys	Arg	Phe	Phe	Phe	Asn	Ile	Phe	Thr	Arg	Gln	
		35				40					45					50	
	TGC	GAA	GAA	TTT	ATA	TAT	GGG	GGA	TGT	GAA	GGA	AAT	CAG	AAT	CGA	TTT	289
	Cys	Glu	Glu	Phe	Ile	Tyr	Gly	Gly	Cys	Glu	Gly	Asn	Gln	Asn	Arg	Phe	
				55						60					65		
20	GAA	AGT	CTG	GAA	GAG	TGC	AAA	AAA	ATG	TGT	ACA	AGA	GAT	AAT	GCA	AAC	337
	Glu	Ser	Leu	Glu	Glu	Cys	Lys	Lys	Met	Cys	Thr	Arg	Asp	Asn	Ala	Asn	
				70					75					80			
	AGG	ATT	ATA	AAG	ACA	ACA	CTG	CAG	CAA	GAA	AAG	CCA	GAT	TTC	TGC	TTT	385
25	Arg	Ile	Ile	Lys	Thr	Thr	Leu	Gln	Gln	Glu	Lys	Pro	Asp	Phe	Cys	Phe	
			85					90					95				
	TTG	GAA	GAG	GAT	OCT	GGA	ATA	TGT	CGA	GGT	TAT	ATT	ACC	AGG	TAT	TTT	433
	Leu	Glu	Glu	Asp	Pro	Gly	Ile	Cys	Arg	Gly	Tyr	Ile	Thr	Arg	Tyr	Phe	
		100					105					110					
	TAT	AAC	AAT	CAG	ACA	AAA	CAG	TGT	GAA	AGG	TTC	AAG	TAT	GGT	GGA	TGC	481
30	Tyr	Asn	Asn	Gln	Thr	Lys	Gln	Cys	Glu	Arg	Phe	Lys	Tyr	Gly	Gly	Cys	
		115				120					125					130	
	CTG	GGC	AAT	ATG	AAC	AAT	TTT	GAG	ACA	CTC	GAG	GAA	TGC	AAG	AAC	ATT	529
	Leu	Gly	Asn	Met	Asn	Asn	Phe	Glu	Thr	Leu	Glu	Glu	Cys	Lys	Asn	Ile	
					135					140					145		
35	TGT	GAA	GAT	GGT	CCG	AAT	GGT	TTC	CAG	GTG	GAT	AAT	TAT	GGT	ACC	CAG	577
	Cys	Glu	Asp	Gly	Pro	Asn	Gly	Phe	Gln	Val	Asp	Asn	Tyr	Gly	Thr	Gln	
				150				155						160			
	CTC	AAT	GCT	GTT	AAC	AAC	TCC	CTG	ACT	CCG	CAA	TCA	ACC	AAG	GTT	CCC	625
40	Leu	Asn	Ala	Val	Asn	Asn	Ser	Leu	Thr	Pro	Gln	Ser	Thr	Lys	Val	Pro	

28

	AGC CTT TTT GAA TTC CAC GGT CCC TCA TGG TGT CTC ACT CCA GCA GAT	673
	Ser Leu Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp	
	180 185 190	
	AGA GGA TTG TGT CGT GCC AAT GAG AAC AGA TTC TAC TAC AAT TCA GTC	721
5	Arg Gly Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn Ser Val	
	195 200 205 210	
	ATT GGG AAA TGC CGC CCA TTT AAG TAC TCC GGA TGT GGG GGA AAT GAA	769
	Ile Gly Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly Asn Glu	
	215 220 225	
10	AAC AAT TTT ACT AGT AAA CAA GAA TGT CTG AGG GCA TGC AAA AAA GGT	817
	Asn Asn Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly	
	230 235 240	
	TTC ATC CAA AGA ATA TCA AAA GGA GGC CTA ATT AAA ACC AAA AGA AAA	865
15	Phe Ile Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys Arg Lys	
	245 250 255	
	AGA AAG AAG CAG AGA GTG AAA ATA GCA TAT GAA GAA ATT TTT GTT AAA	913
	Arg Lys Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe Val Lys	
	260 265 270	
	AAT ATG TGAGTGGAC	928
20	Asn Met	
	275	

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 304 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Met	Ile	Tyr	Thr	Met	Lys	Lys	Val	His	Ala	Leu	Trp	Ala	Ser	Val	Cys
30	-28		-25				-20						-15		
Leu	Leu	Leu	Asn	Leu	Ala	Pro	Ala	Pro	Leu	Asn	Ala	Asp	Ser	Glu	Glu
	-10				-5							1			
Asp	Glu	Glu	His	Thr	Ile	Ile	Thr	Asp	Thr	Glu	Leu	Pro	Pro	Leu	Lys
5			10				15					20			
35	Leu	Met	His	Ser	Phe	Cys	Ala	Phe	Lys	Ala	Asp	Asp	Gly	Pro	Cys
		25					30						35		
Ala	Ile	Met	Lys	Arg	Phe	Phe	Phe	Asn	Ile	Phe	Thr	Arg	Gln	Cys	Glu
		40					45						50		

29

Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn Arg Phe Glu Ser
 55 60 65
 Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn Ala Asn Arg Ile
 70 75 80
 5 Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe Cys Phe Leu Glu
 85 90 95 100
 Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn
 105 110 115
 10 Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly
 120 125 130
 Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu
 135 140 145
 Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly Thr Gln Leu Asn
 150 155 160
 15 Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys Val Pro Ser Leu
 165 170 175 180
 Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp Arg Gly
 185 190 195
 20 Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn Ser Val Ile Gly
 200 205 210
 Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly Asn Glu Asn Asn
 215 220 225
 Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly Phe Ile
 230 235 240
 25 Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys Arg Lys Arg Lys
 245 250 255 260
 Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe Val Lys Asn Met
 265 270 275

(2) INFORMATION FOR SEQ ID NO: 10:

- 30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 234 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 35 (ii) MOLECULE TYPE: DNA
 (iii) HYPOTHETICAL: NO
 (iii) ANTI-SENSE: NO

30

(vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(ix) FEATURE:

(A) NAME/KEY: CDS

5 (B) LOCATION: 76..234

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 76..222

(ix) FEATURE:

10 (A) NAME/KEY: mat_peptide

(B) LOCATION: 223..234

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

	GAATTCATTC AAGAATAGTT CAAACAAGAA GATTACAAAC TATCAATTTC ATACACAATA	60
15	TAAACGATTA AAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC GGA	111
	Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly	
	-49 -45 -40	
	TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG ATT	159
	Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile	
	-35 -30 -25	
20	CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC GTC	207
	Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val	
	-20 -15 -10	
	GCC ATG GCT AAG AGA GAT TCT GAG GAA	234
25	Ala Met Ala Lys Arg Asp Ser Glu Glu	
	-5 1	

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53 amino acids

30 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

	Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala	
	-49 -45 -40 -35	
35	Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser	
	-30 -25 -20	
	Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Lys	
	-15 -10 -5	

31

Arg Asp Ser Glu Glu
1

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
5 (A) LENGTH: 190 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- 10 (iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic
- (ix) FEATURE:
15 (A) NAME/KEY: CDS
(B) LOCATION: 17..190
- (ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 17..178
- 20 (ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 179..190

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

25	GAATTCAAAC TAAAAA ATG AAG CTT AAA ACT GTA AGA TCT GCG GTC CTT	49
	Met Lys Leu Lys Thr Val Arg Ser Ala Val Leu	
	-54 -50 -45	
	TOG TCA CTC TTT GCA TCG CAG GTC CTA GGT CAA OCA GTC ACT GGC GAT	97
	Ser Ser Leu Phe Ala Ser Gln Val Leu Gly Gln Pro Val Thr Gly Asp	
	-40 -35 -30	
30	GAA TCA TCT GTT GAG ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC	145
	Glu Ser Ser Val Glu Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn	
	-25 -20 -15	
	ACC ACT TTG GCT AAC GTC GCC ATG GCT AAG AGA GAT TCT GAG GAA	190
35	Thr Thr Leu Ala Asn Val Ala Met Ala Lys Arg Asp Ser Glu Glu	
	-10 -5 1	

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids

32

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

5 Met Lys Leu Lys Thr Val Arg Ser Ala Val Leu Ser Ser Leu Phe Ala
-54 -50 -45 -40
Ser Gln Val Leu Gly Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
-35 -30 -25
10 Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
-20 -15 -10
Val Ala Met Ala Lys Arg Asp Ser Glu Glu
-5 1

(2) INFORMATION FOR SEQ ID NO: 14:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20 (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATTGCTGOC ATGGTACTTT CAGAAGG

27

(2) INFORMATION FOR SEQ ID NO: 15:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CAACCAATAG ACAAGGTTAA AGAAGGCCTA CAGCATGATT ACGATACAGA GATCTTGGAG

60

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCAAGATCTC TGTATOGTAA TCATGCIGTA GGCTTCTTT ACGGTGTCT ATTGGTTGGG 60
CC 62

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GTACCAAAT AATGAACTG AAACTGTAA GATCTGGGT CCITTOGTCA CTCITTCAT 60
CTCAGGTCT TGGCCAACA ATAGACA 87

25 (2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

34

OGGTTGTCTA TTGGTTGGCC AAGGACCTGA GATGCAAAGA GTGACGAAAG GACGSCAGAT 60
CTTACAGTTT TCAGTTTCTA TATTTTG 87

(2) INFORMATION FOR SEQ ID NO: 19:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- 10 (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TAAAGTGGC

9

(2) INFORMATION FOR SEQ ID NO: 20:

- 15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CATGGGAGCG

10

25 (2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

35

CTTGGCCAAC CATOGAAATT GAAACCAG

28

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

- 10 (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CTAGCTGGTT TCAATTTOGA TGGTIGGC

28

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 88 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

- 20 (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

AATTCAAAC TAAAAATGAA GCTTAAAC TGAAGATCTG OGGTCTTTC GTCACTCTTT

60

GCATGCAGG TCTAGGTCA ACCAGTCA

88

25 (2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

WO 95/02059

PCT/DK94/00281

36

CIGGTTGACC TAGGAOCTGC GATGCAAAGA GTGACGAAAG GACCGCAGAT CTTACAGTTT 60

TAAGCTTCAT TTTTAGTTT G 81

CLAIMS

1. A DNA construct comprising the following sequence

5'-P-SP-(LP)_n-PS-HP-3'

wherein

- 5 P is a promoter sequence,
SP is a DNA sequence encoding the yeast aspartic protease 3 (YAP3) signal peptide,
LP is a DNA sequence encoding a leader peptide,
n is 0 or 1,
- 10 PS is a DNA sequence encoding a peptide defining a yeast processing site, and
HP is a DNA sequence encoding a polypeptide which is heterologous to a selected host organism.
2. A DNA construct according to claim 1, wherein the promoter
15 sequence is selected from the Saccharomyces cerevisiae MFα1, TPI, ADH, BAR1 or PGK promoter, or the Schizosaccharomyces pombe ADH promoter.
3. A DNA construct according to claim 1, wherein the YAP3 signal peptide is encoded by the following DNA sequence
- 20 ATG AAA CTG AAA ACT GTA AGA TCT GCG GTC CTT TCG TCA CTC TTT GCA
TCT CAG GTC CTT GGC (SEQ ID No:1)
- or a suitable modification thereof encoding a peptide with a high degree of homology to the YAP3 signal peptide.
4. A DNA construct according to claim 1, wherein n is 1.
- 25 5. A DNA construct according to claim 5, wherein the leader peptide is a yeast MFα1 leader peptide or a synthetic leader peptide.

6. A DNA construct according to claim 1, wherein PS is a DNA sequence encoding Lys-Arg, Arg-Lys, Lys-Lys, Arg-Arg or Ile-Glu-Gly-Arg.
7. A DNA construct according to claim 1, wherein the
5 heterologous polypeptide is selected from the group consisting of aprotinin, tissue factor pathway inhibitor or other protease inhibitors, insulin or insulin precursors, human or bovine growth hormone, interleukin, glucagon, glucagon-like peptide 1, tissue plasminogen activator, transforming growth factor α or
10 β , platelet-derived growth factor, enzymes, or a functional analogue thereof.
8. A DNA construct according to claim 1, which further comprises a transcription termination sequence.
9. A DNA construct according to claim 8, wherein the
15 transcription termination sequence is the TPI terminator.
10. A recombinant expression vector comprising a DNA construct according to any of claims 1-9.
11. A cell transformed with a vector according to claim 10.
12. A cell according to claim 11, which is a fungal cell.
- 20 13. A cell according to claim 12, which is a yeast cell.
14. A cell according to claim 13, which is a cell of Saccharomyces, Schizosaccharomyces, Kluyveromyces, Hansenula or Yarrowia.
15. A cell according to claim 14, which is a cell of
25 Saccharomyces cerevisiae or Schizosaccharomyces pombe.

16. A method of producing a heterologous polypeptide, the method comprising culturing a cell which is capable of expressing a heterologous polypeptide and which is transformed with a DNA construct according to any of claims 1-9 in a
5 suitable medium to obtain expression and secretion of the heterologous polypeptide, after which the heterologous polypeptide is recovered from the medium.

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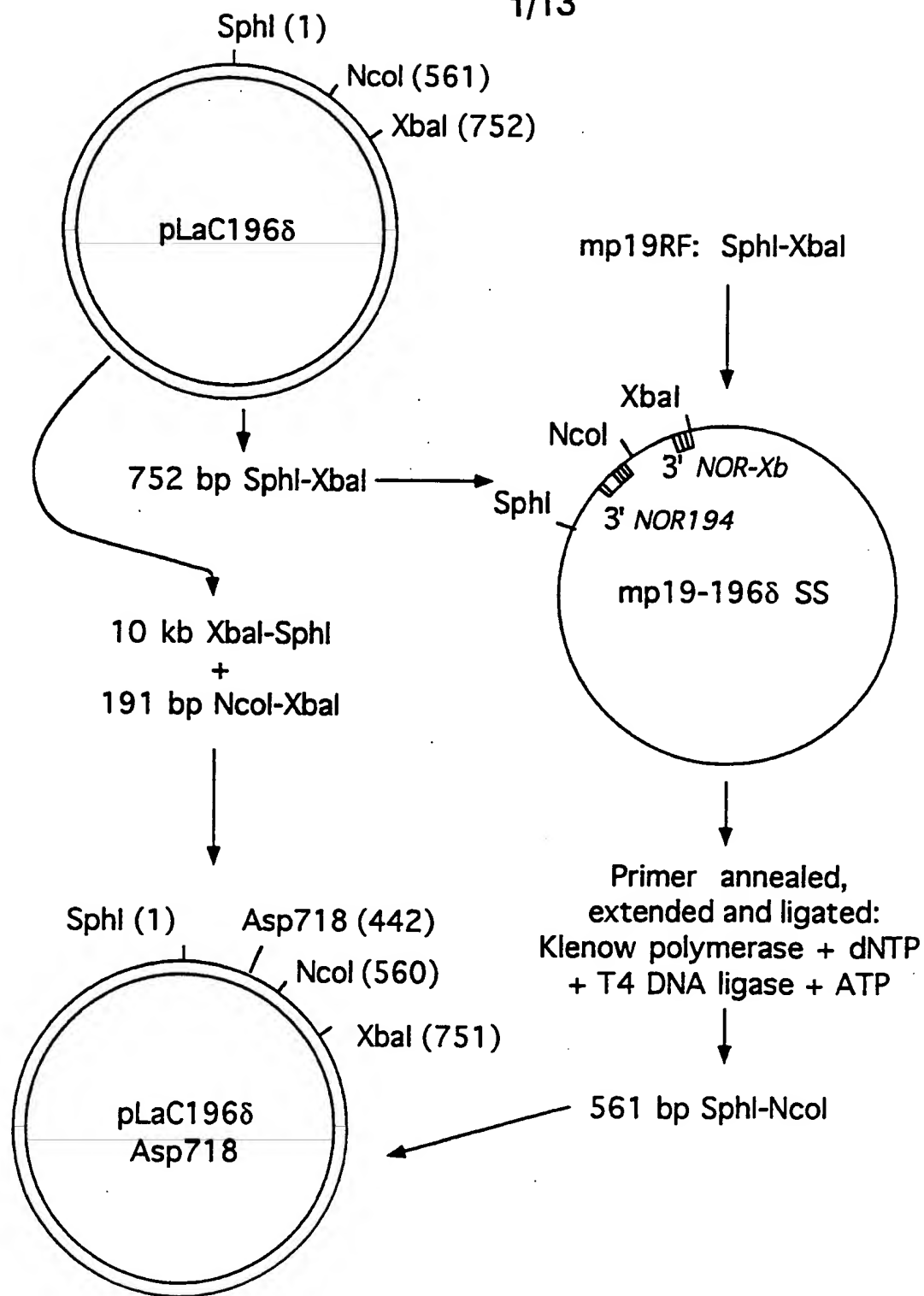


Fig. 1a

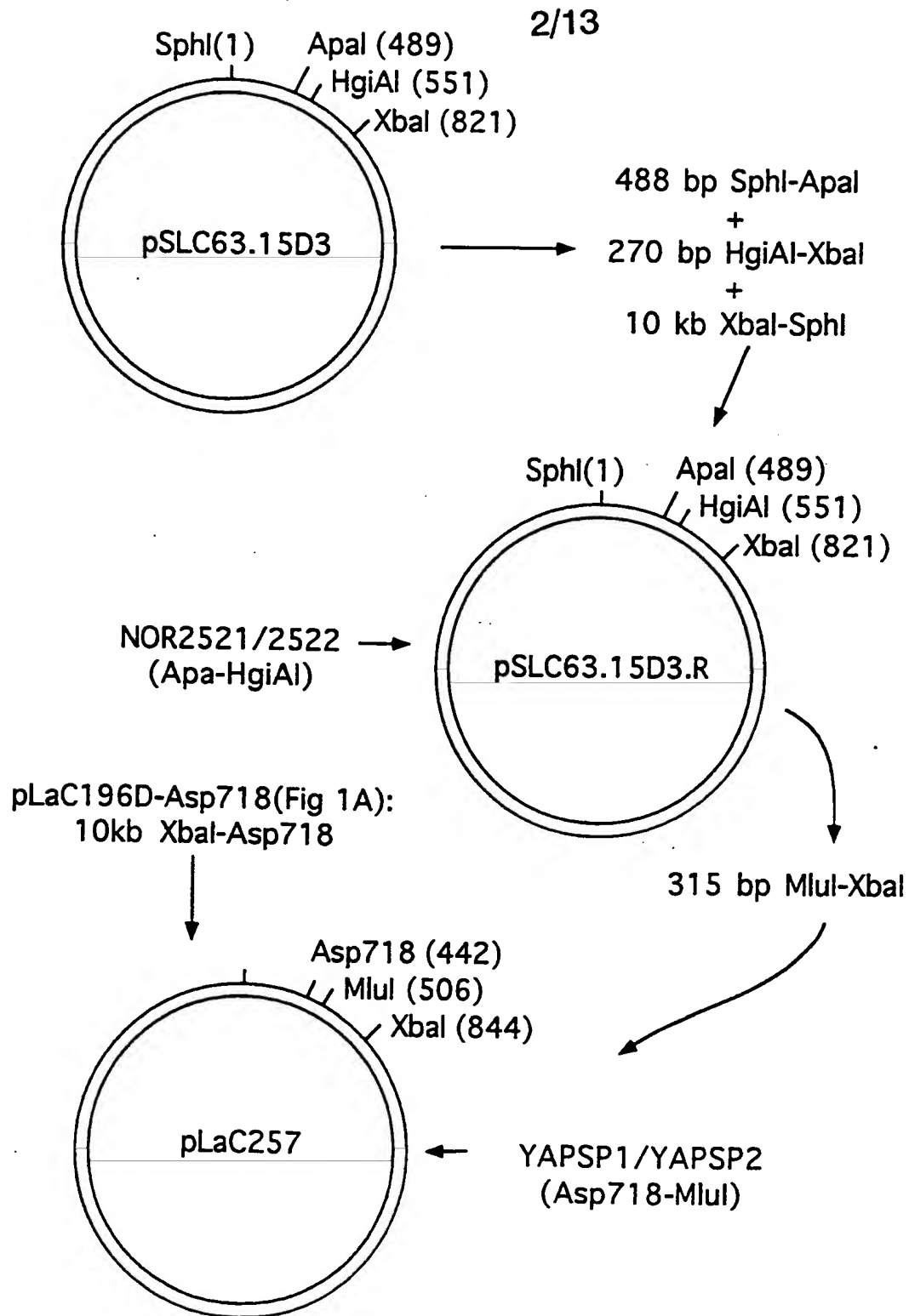


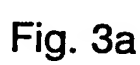
Fig. 1b

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[illegible]

Fig. 2

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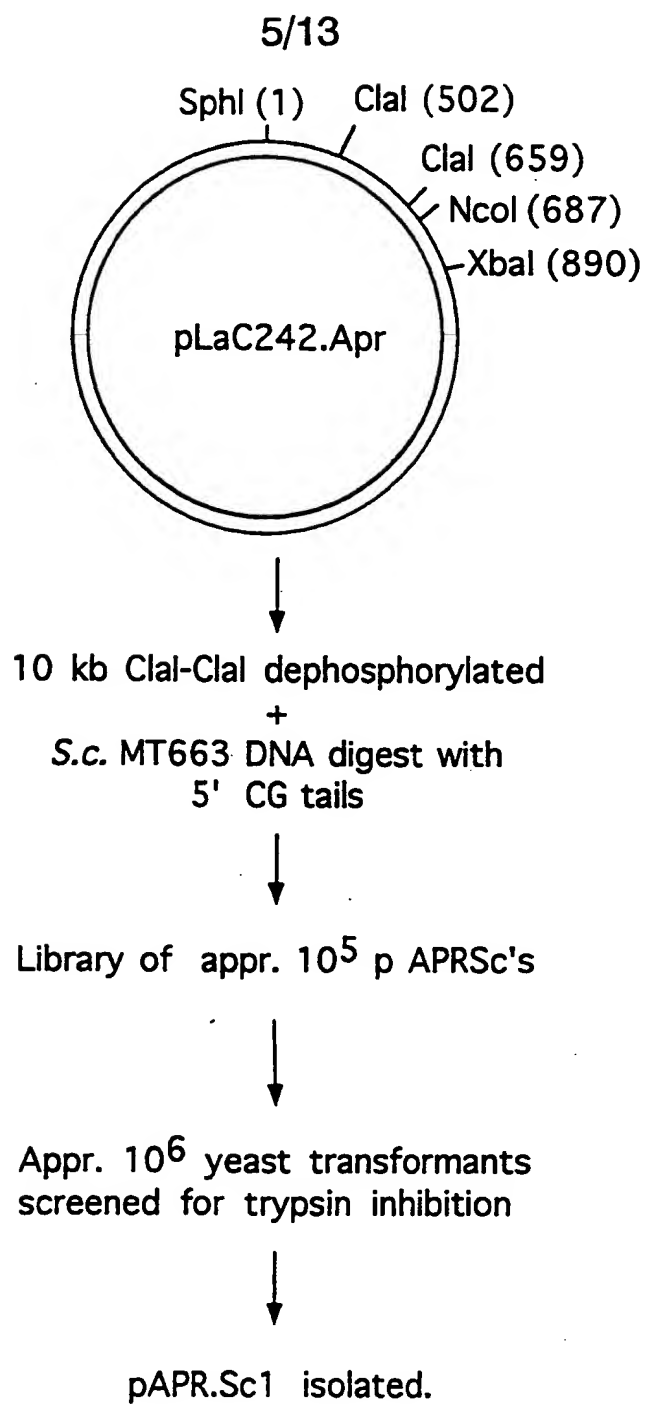


Fig. 3b

[illegible]

Fig. 4

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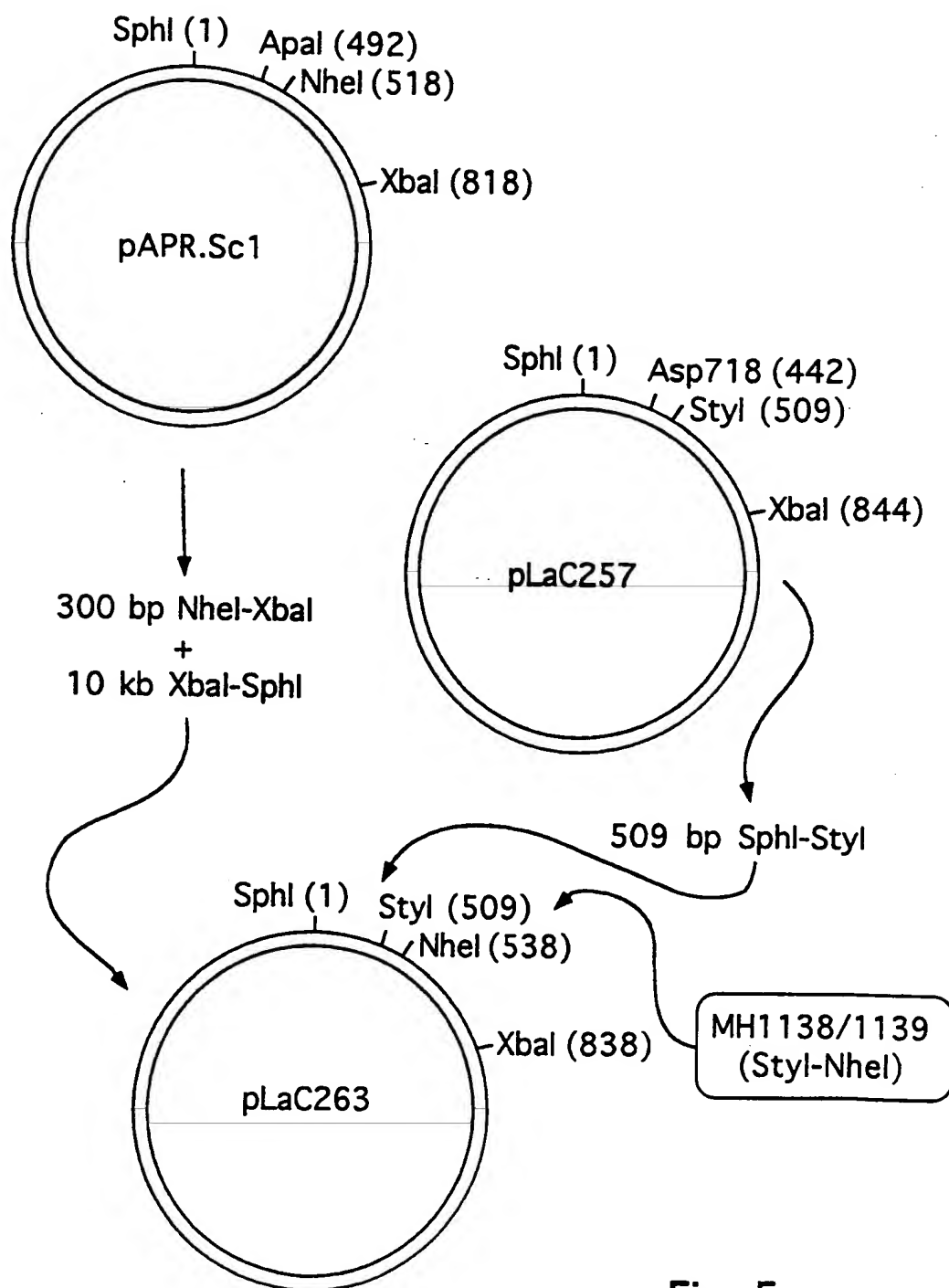


Fig 5

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[illegible]

Fig. 6

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[illegible]

Fig. 7a

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TGT	GAA	GAT	GGT	CCG	AAT	GGT	TTC	CAG	GTG	GAT	AAT	TAT	GGT	ACC	CAG	577
Cys	Glu	Asp	Gly	Pro	Asn	Gly	Phe	Gln	Val	Asp	Asn	Tyr	Gly	Thr	Gln	
			150					155					160			
KpnI																
CTC	AAT	GCT	GTT	AAC	AAC	TCC	CTG	ACT	CCG	CAA	TCA	ACC	AAG	GTT	CCC	625
Leu	Asn	Ala	Val	Asn	Asn	Ser	Leu	Thr	Pro	Gln	Ser	Thr	Lys	Val	Pro	
		165					170					175				
HpaI																
AGC	CTT	TTT	GAA	TTC	CAC	GGT	CCC	TCA	TGG	TGT	CTC	ACT	CCA	GCA	GAT	673
Ser	Leu	Phe	Glu	Phe	His	Gly	Pro	Ser	Trp	Cys	Leu	Thr	Pro	Ala	Asp	
		180				185					190					
EcoRI																
AGA	GGA	TTG	TGT	CGT	GCC	AAT	GAG	AAC	AGA	TTC	TAC	TAC	AAT	TCA	GTC	721
Arg	Gly	Leu	Cys	Arg	Ala	Asn	Glu	Asn	Arg	Phe	Tyr	Tyr	Asn	Ser	Val	
		195			200				205						210	
ΔEcoRV																
ATT	GGG	AAA	TGC	CGC	CCA	TTT	AAG	TAC	TCC	GGA	TGT	GGG	GGA	AAT	GAA	769
Ile	Gly	Lys	Cys	Arg	Pro	Phe	Lys	Tyr	Ser	Gly	Cys	Gly	Gly	Asn	Glu	
				215					220					225		
BspMII																
AAC	AAT	TTT	ACT	AGT	AAA	CAA	GAA	TGT	CTG	AGG	GCA	TGC	AAA	AAA	GGT	817
Asn	Asn	Phe	Thr	Ser	Lys	Gln	Glu	Cys	Leu	Arg	Ala	Cys	Lys	Lys	Gly	
			230					235					240			
SpeI																
SphI																
TTC	ATC	CAA	AGA	ATA	TCA	AAA	GGA	GGC	CTA	ATT	AAA	ACC	AAA	AGA	AAA	865
Phe	Ile	Gln	Arg	Ile	Ser	Lys	Gly	Gly	Leu	Ile	Lys	Thr	Lys	Arg	Lys	
		245					250					255				
StuI																
AGA	AAG	AAG	CAG	AGA	GTG	AAA	ATA	GCA	TAT	GAA	GAA	ATT	TTT	GTT	AAA	913
Arg	Lys	Lys	Gln	Arg	Val	Lys	Ile	Ala	Tyr	Glu	Glu	Ile	Phe	Val	Lys	
		260				265					270					
SalI																
AAT	ATG	TGAGTCGAC														928
Asn	Met															
		275														

Fig. 7b

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EcoRI
5361 GAATTCATTCAAGAATAGTTCAAACAAGAAGATTACAACTATCAATTTCATACACAAT
5420 ATAAACGATTAAAAGAATGAAGGCTGTTTTCTTGTTTTGTCCTTGATCGGATTCTGCT
MetLysAlaValPheLeuValLeuSerLeuIleGlyPheCys
-----spx3 signal peptide-----

PfIMI BspEI BclI
5479 GGGCCCAACCAGTCACTGGCGATGAATCATCTGTTGAGATTCCGGAAGAGTCTCTGATC
TrpAlaGlnProValThrGlyAspGluSerSerValGluIleProGluGluSerLeuIle
-----+212 leader -----

5438 ATCGCTGAAAACACCACTTTGGCTAACGTCGCCATGGCTAAGAGAGATTCTGAGGAA
IleAlaGluAsnThrThrLeuAlaAsnValAlaMetAlaLysArgAspSerGluGlu--
-----TFPI --
Kex2

Fig. 8a

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```

EcoRI          HindIII      BglII
5361 GAATTCAAAC TAAAAAATGAAGCTTAAACTGTAAGATCTGCGGTCCTTTTCGTCAC TCT
      MetLysLeuLysThrValArgSerAlaValLeuSerSerLeu
      -----Yap3 signal peptide-----

          AvrII      PflMI
5420 TTGCATCGCAGGTCCTAGGTCAACCAGTCACTGGCGATGAATCATCTGTTGAGATTCCG
      PheAlaSerGlnValLeuGlyGlnProValThrGlyAspGluSerSerValGluIlePro
      -----+212 leader-----

          BclI
5479 GAAGAGTCTCTGATCATCGCTGAAAACACCACTTTGGCTAACGTCGCCATGGCTAAGAG
      GluGluSerLeuIleIleAlaGluAsnThrThrLeuAlaAsnValAlaMetAlaLys
      +-----+

          NcoI
5538 AGATTCTGAGGAA--
      ArgAspSerGluGlu--
      +++-TFPI --
```

Fig. 8b

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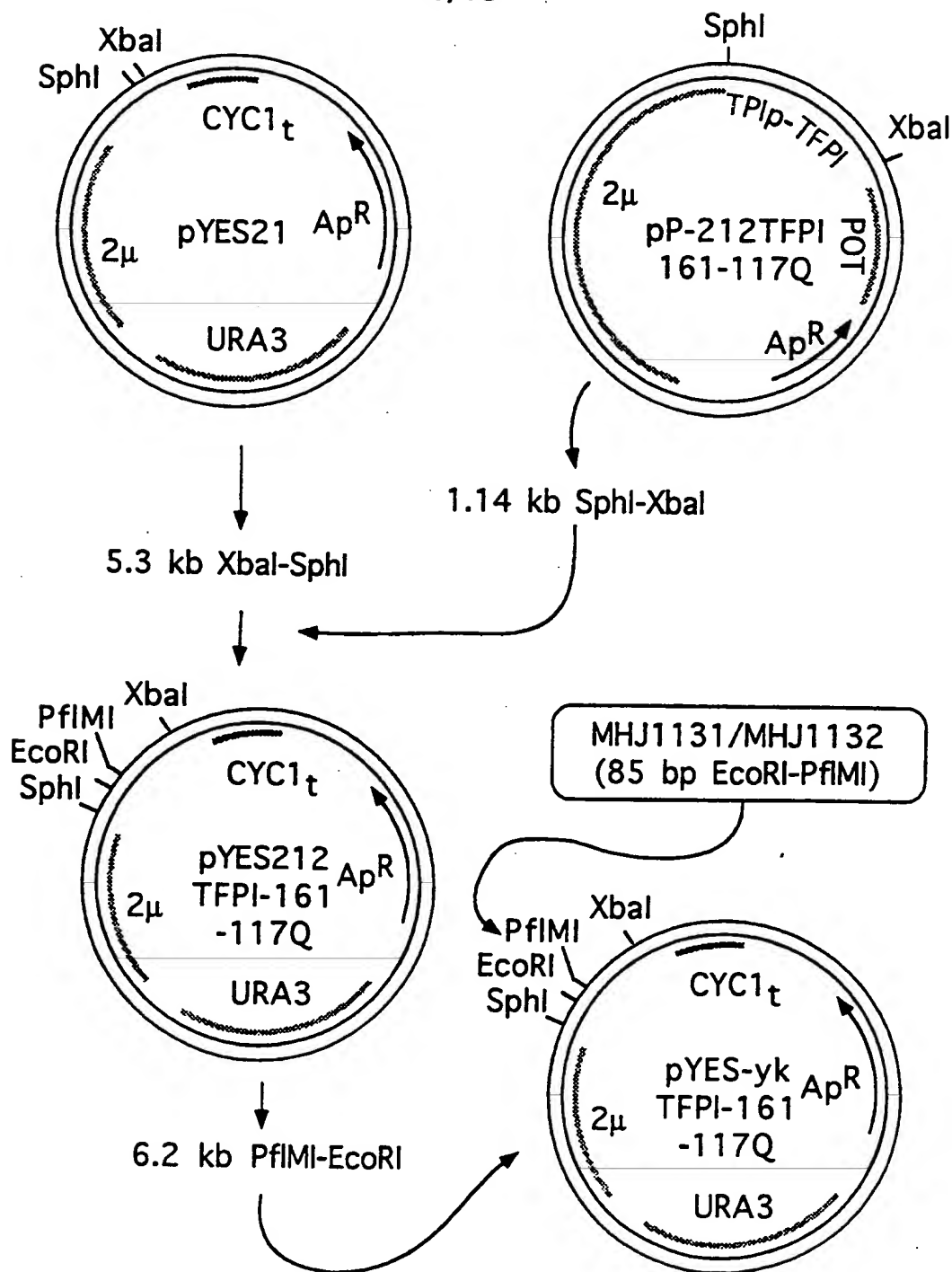


Fig. 9

1
INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00281

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/81, C12N 15/62
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPIL, US PATENTS FULLTEXT DATABASES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 8702670 (MACKAY, VIVIAN, L), 7 May 1987 (07.05.87)	1-16
X	JOURNAL OF CELLULAR BIOCHEMISTRY/SUPPLEMENT, Volume 12, 1988: Suppl. O. Part B, Susan K. Welch et al, "A unique structural domain of a yeast aspartyl protease directs protein secre- tion", page 287	1-16

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

- | | |
|---|--|
| <ul style="list-style-type: none"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed | <ul style="list-style-type: none"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family |
|---|--|

Date of the actual completion of the international search

Date of mailing of the international search report

24 October 1994

27 -10- 1994

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01/10/94

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		AU-A- 6543286	19/05/87
		AU-A- 7400391	18/07/91
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		EP-A,B- 0220689	06/05/87
		SE-T3- 0220689	
		EP-A- 0243465	04/11/87
		JP-T- 63501614	23/06/88